FINAL REPORT

Development of Biomarkers for Assessing In Situ RDX Biodegradation Potential

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14. ABSTRACT

This project addresses the problem of widespread RDX contamination at DoD sites. The objective was to identify the microorganisms responsible for the biodegradation of RDX in complex, mixed culture samples through the application of stable isotope probing (SIP). This approach identifies microorganisms responsible for a particular function without cultivation therefore, RDX degradation can ultimately be studied under conditions more similar to those at contaminated sites. Additionally, only active organisms are targeted. For this, RDX degrading microcosms were exposed to labeled RDX and after an incubation period DNA was extracted ultracentrifuged (to separate the labeled nucleic acid from the unlabeled background nucleic acid) and finally molecular analysis steps (terminal restriction fragment length polymorphism TRFLP, 16S rRNA gene sequencing) were performed to identify the organisms responsible for label uptake from RDX. Two RDX concentrations were examined (10 and 20 ppm), however only the higher concentration resulted in a significant SIP signal. In these ultracentrifugation fractions only one TRFLP fragment (260 bp) showed a reliable trend of label uptake. Specifically, this fragment was of higher relative abundance in the heavier fractions from labeled samples compared to the heavier fractions from the unlabeled control samples. Partial 16S rRNA gene sequencing indicated the organisms represented by fragment 260 bp belonged to either the Sphingobacteria or the Acidobacteria. In conclusion, the proof-of-concept was achieved and the methods could be applied to other RDX transforming cultures or environmental samples to determine additional RDX degraders in complex samples and thus biomarkers for assessing the feasibility of natural attenuation.

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2010 LIMITED SCOPE PROJECT
FINAL REPORT

Development of Biomarkers for Assessing *In Situ* RDX Biodegradation Potential

Project Number: ER 1606

Submitted to: Dr. Andrea Leeson

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LIST OF ACRONYMS

SERDP	Strategic Environmental Research and Development Program
BD	Buoyant density
CsCl	Cesium chloride
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
HMX	high melting explosive, octahydro-1,3,5,7-tetranitro-1,3,5.7-tetrazocine
HPLC	High performance liquid chromatography
LB	Luria Bertani broth
MNX	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
SIP	Stable isotope probing
RDX	Royal demolition explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA	Ribonucleic acid
TNX	Hexahydro-1,3,5-trinitroso -1,3,5-triazine
TRFLP	Terminal restriction fragment length polymorphism

KEYWORDS

RDX, SIP, stable isotope probing, *Sphingobacteria, Acidobacteria,* TRFLP

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1.0. ABSTRACT

This project addresses the problem of widespread RDX contamination at DoD sites. The objective was to identify the microorganisms responsible for the biodegradation of RDX in complex, mixed culture samples through the application of stable isotope probing (SIP). This approach identifies microorganisms responsible for a particular function without cultivation; therefore, RDX degradation can ultimately be studied under conditions more similar to those at contaminated sites. Additionally, only active organisms are targeted. For this, RDX degrading microcosms were exposed to labeled RDX and after an incubation period DNA was extracted, ultracentrifuged (to separate the labeled nucleic acid from the unlabeled background nucleic acid) and finally molecular analysis steps (terminal restriction fragment length polymorphism, TRFLP, 16S rRNA gene sequencing) were performed to identify the organisms responsible for label uptake from RDX. Two RDX concentrations were examined (10 and 20 ppm), however, only the higher concentration resulted in a significant SIP signal. In these ultracentrifugation fractions only one TRFLP fragment (260 bp) showed a reliable trend of label uptake. Specifically, this fragment was of higher relative abundance in the heavier fractions from labeled samples compared to the heavier fractions from the unlabeled control samples. Partial 16S rRNA gene sequencing indicated the organisms represented by fragment 260 bp belonged to either the *Sphingobacteria* or the *Acidobacteria*. In conclusion, the proof-of-concept was achieved and the methods could be applied to other RDX transforming cultures or environmental samples to determine additional RDX degraders in complex samples and thus biomarkers for assessing the feasibility of natural attenuation.

2.0. PROJECT OBJECTIVES

2.1. Overall Objective

To develop a better understanding of the microorganisms responsible for the biodegradation of RDX in complex, mixed culture samples through the application of the molecular method stable isotope probing (SIP). This overall objective was addressed with two overall tasks, as follows.

- Microcosm studies were conducted with ten different soils to screen for RDX biodegradation activity.

- In one soil type, the proof-of-concept that SIP can be used to identify RDX degraders in mixed community samples was investigated. This involved:

    - Method development, including the appropriate concentration of RDX needed to result in a detectable signal for SIP.

    - The determination of the identity (16S rRNA gene sequences) of the microorganisms responsible for RDX transformation.

2.2. Addressing the SERDP Statement of Need (SON)

This work was funded under the SON ERSON-08-02 entitled "Improved understanding of the biological degradation of nitroamines in the environment". The project focused on the development and application of methods required for an increased understanding of the biological degradation of nitroamines. The work specifically targeted RDX biodegradation in mixed community, complex samples as these are more representative of the transformation that would occur *in situ* at contaminated sites.

2.3. Statement Regarding Success of the Project and Future Work

This Limited Scope project can be considered successful because laboratory protocols were developed to investigate RDX degraders in complex samples and these methods can now be applied to study RDX degraders in a variety of samples. Therefore, the work forms a basis for future work involving the development of additional biomarkers for use in assessing the potential for RDX biodegradation at contaminated sites. If funding were available, our laboratory is perfectly poised to apply this methodology to determine the microorganisms responsible for RDX removal in a larger number of RDX degrading, complex, mixed culture samples or microcosms. A more extensive follow-on project would facilitate the identification of a larger number of RDX degrading species, enabling the creation of a large library of RDX biomarkers for use in monitoring natural attenuation at contaminated sites.

3.0. BACKGROUND

3.1. Environmental Issue Addressed

The project addresses the problem of the widespread contamination of DoD sites with explosives. Such contamination has been associated with manufacturing and load-assemble-package (LAP) processes performed during or after World War II and the Korean Conflict. Remediation of many of these sites has been initiated since the early 1980s, however many still have groundwater contaminated with nitroaromatics. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is the most problematic of these because of its high frequency of use, high solubility, recalcitrance to abiotic and biotic processes and toxicity.

3.2. Regulatory Environment

The U.S. Environmental Protection Agency (EPA) has established health advisory (HA) levels for RDX in drinking water (2 μg/L). The health advisory indicates the potential threat that this chemical poses to humans and other organisms.

3.3. Previous Research on RDX Biodegradation

Previous research has illustrated the susceptibility of RDX (Figure 1) to biodegradation in the laboratory. For example, under aerobic conditions, *Stenotrophomonas maltophilia* PB1, *Rhodococcus* sp. strain DN22 and *Rhodococcus rhodochrous* strain 11Y can use RDX as the sole source of nitrogen for growth (Binks et al., 1995; Coleman et al., 1998; Fournier et al., 2002; Seth-Smith et al., 2002). Pathways of RDX degradation by these organisms are discussed below.

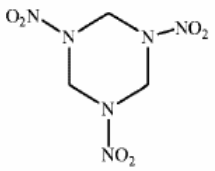

Figure 1. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)

During RDX degradation by *S. maltophilia* PB1, three moles of nitrogen per mole of RDX are utilized, and a metabolite tentatively identified as methylene-*N*-(hydroxymethyl)-hydroxylamone-*N′*-(hydroxymethyl)nitroamine (Figure 2) is formed. When this organism was grown within a mixed culture the metabolite did not accumulate, thus the authors concluded that other organisms in the culture could metabolize this compound (Binks et al., 1995). Notably, RDX was not degraded by *S. maltophilia* PB1 in the presence of an additional nitrogen source (NH_4NO_3) (Binks et al., 1995). Further, RDX degradation required the addition of a carbon source, indicating the organism was unable to use RDX as a sole carbon source (Binks et al., 1995).

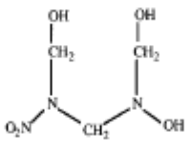

Figure 2. Methylene-*N*-(hydroxymethyl)-hydroxylamone-*N′*-(hydroxymethyl)nitroamine, the metabolite produced during RDX degradation by *Stenotrophomonas maltophilia* PB1

Rhodococcus sp. strain DN22 converts RDX to nitrite, nitrous oxide, ammonia, formaldehyde and an unidentified dead end product (Fournier et al., 2002), later identified as 4-nitro-2,4-

diazabutanal (NDAB) (Figure 3) (Bhushan et al., 2003). Growth yields of *Rhodococcus* sp. strain DN22 indicted that three of the six RDX nitrogen atoms are incorporated into biomass (Coleman et al., 1998). Additional studies with this organism indicated that two ring nitrogen atoms and two ring carbon atoms were incorporated in the metabolite (Fournier et al., 2002). When this organism was grown with other N sources ($NaNO_3$, $NaNO_2$, $(NH_4)_2SO_4$ or glutamine), only $(NH_4)_2SO_4$ significantly inhibited RDX degradation (Coleman et al., 1998). *Rhodococcus* sp. strain DN22 could not grow in media with RDX as the sole source of carbon or of carbon and nitrogen (Coleman et al., 1998). RDX degradation by *Rhodococcus* sp. strain DN22 likely involves a plasmid-borne cytochrome P450 enzyme (Bhushan et al., 2003; Coleman et al., 2002). The proposed pathway for RDX biotransformation involves cytochrome P450 catalyzing the sequential transfer of two single electrons to RDX (Figure 3) (Bhushan et al., 2003). The first electron causes denitration to create compound I, and the second electron causes a second denitration to form compound II. Compound II is unstable in water and is hydrolyzed to form compound III. Following this, the spontaneous decomposition of compound III produces NDAB (Bhushan et al., 2003).

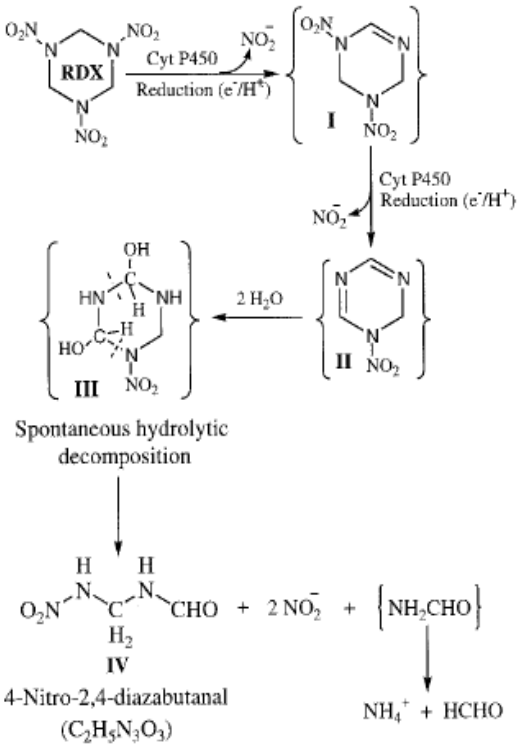

Figure 3. Proposed pathway for RDX biotransformation by *Rhodococcus* sp. strain DN22. Structures in parenthesis were not detected by these researchers (Bhushan et al., 2003).

RDX degradation in *Rhodococcus rhodochrous* strain 11Y also involves initial denitration followed by spontaneous ring cleavage (Seth-Smith et al., 2002) and formation of NDAB (Thompson et al., 2005a). The gene responsible for RDX degradation in *R. rhodochrous* has been identified. This gene, *xplA*, is constitutively expressed and encodes for an enzyme with homology to a cytochrome P450 (Seth-Smith et al., 2002). The gene was recently cloned into *Arabidopsis thaliana,* and the plant could consequently detoxify RDX from liquid media (Rylott et al., 2006). *R. rhodochrous* strain 11Y uses three moles of nitrogen per mole of RDX and cannot use RDX as a sole source of carbon (Seth-Smith et al., 2002).

In significant contrast to the organisms discussed above the bacteria, *Williamsia* sp. KTR4 and *Gordonia* sp. KTR9, can use RDX as a sole carbon and nitrogen source to support growth (Thompson et al., 2005a). These organisms are able to mineralize RDX when used as a source of carbon, nitrogen, or carbon and nitrogen (Thompson et al., 2005a). Both organisms were able to transform RDX faster as a nitrogen source (half life of 0.89 d and 0.63 d for KTR4 and KTR 9), than as a carbon source (1.14 d and 11.20 d for KTR4 and KTR 9) or as a carbon and nitrogen source (1.16 d and 1.07 d for KTR4 and KTR 9) (Thompson et al., 2005a). In the presence of $(NH_4)_2SO_4$, RDX degradation was greatly inhibited in KTR9, but has little effect on RDX degradation by KTR4 (Thompson et al., 2005a). These cultures also transformed RDX to nitrite, formaldehyde and the dead-end product NDAB. NDAB is susceptible to biological transformation by a white-rot fungus (*Phanerochaete chrysosporium*) and by a methylotrophic bacterium (*Methylobacterium* sp. strain JS178) (Fournier et al., 2005; Fournier et al., 2004).

Anaerobic RDX degradation involves at least two degradation pathways (Hawari et al., 2000a; Hawari et al., 2000b). One pathway occurs via the intermediates hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)(McCormick et al., 1981), forming methanol and formaldehyde. The other pathway identified two metabolites (methylenedinitramine and dimethanolnitramine); both rapidly decomposed in water to produce nitroamine and formaldehyde, then nitrous oxide and carbon dioxide (Hawari et al., 2000b). Numerous other investigators have studied RDX anaerobic removal (Adrian and Chow, 2001; Adrian and Arnett, 2004; Beller, 2002; Kitts et al., 1994; Kitts et al., 2000; Young et al., 1997; Zhao et al., 2003b; Zhao et al., 2002; Zhao et al., 2004a; Zhao et al., 2004b), with some microorganisms being able to use RDX as the sole N source (Boopathy et al., 1998; Zhao et al., 2003a).

The above studies all clearly illustrate that RDX biodegradation under laboratory conditions is possible. However, the RDX degrading abilities of these organisms in the field has not yet been determined. To date, the approach to identify RDX degrading microorganisms has been to isolate the microbial strains from the environment, study them in the laboratory and infer potential functions of these microbes in the natural environment. However, microorganisms are exposed to harsher conditions in the environment and it is likely survival under laboratory conditions will not translate to the field. Of particular importance will be the availability of C and N in the field. Further, it is now widely recognized that only a small fraction of microorganisms can be isolated and cultivated in the laboratory (Amann et al., 1995), therefore, it is likely that *in situ* RDX degraders have yet to be identified.

Towards of goal of understanding RDX degradation *in situ,* the work investigated the use of stable isotope probing (SIP) (Figure 4) to identify RDX degrading microorganisms in mixed culture samples. SIP identifies organisms responsible for a particular function without cultivation, therefore, RDX degradation can be studied under conditions imitating those experienced in the field e.g. mixed culture conditions. Additionally, unlike many other molecular approaches, only active organisms are targeted. The method involves sample exposure to a labeled substrate, incubation, nucleic acid extraction, ultracentrifugation to separation the labeled nucleic acid from the unlabeled background nucleic acid, and finally molecular analysis (terminal restriction

fragment length polymorphism, 16S rRNA gene sequencing) to identify the organisms responsible for label uptake.

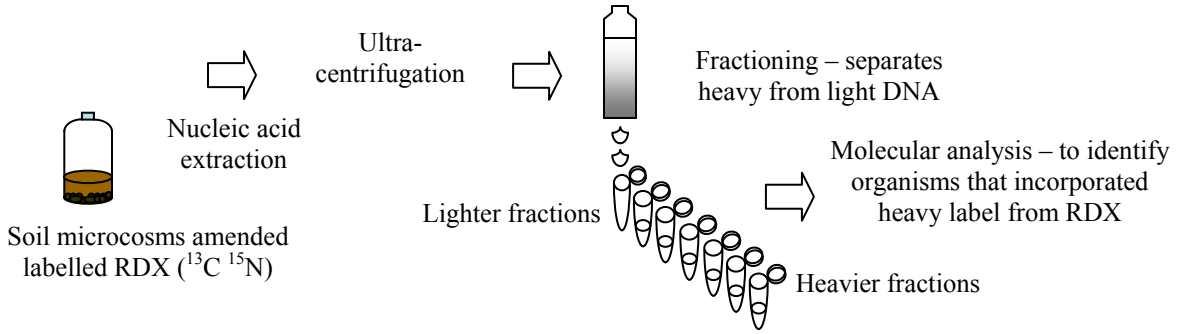

Figure 4. Overview of stable isotope probing (SIP) methods

3.4. Proof-of-Concept

The aim was to determine if SIP could be used to identify RDX degraders in mixed culture samples. This information can ultimately be used to assess the potential for bioremediation and natural attenuation at contaminated sites. The risks associated with the method development involved 1) obtaining microcosms and the experimental conditions necessary for RDX biodegradation, 2) the small increase in DNA buoyant density caused by ^{15}N uptake into DNA resulting in a limited SIP signal, 3) microorganisms degrading RDX in one microcosm sample may not be found in other samples (or contaminated sites) and 4) the limited time available for such a complex project. The work is innovative because at the time this work was initiated, this was one of the first applications of ^{15}N SIP to study RDX biodegradation. Therefore, this was of the first attempts to understand RDX biodegradation in complex, mixed culture samples. The scientific and technical benefits of the project involve the creation of biomarkers (PCR primers targeted to RDX degraders), useful for assessing bioremediation potential at RDX contaminated sites.

4.0. MATERIALS AND METHODS

4.1. Chemicals

4.1. Unlabeled RDX and ring labeled RDX ($^{15}N_3$, $^{13}C_3$; 50% N Labeled) (>99%) dissolved in acetonitrile were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) (Figure 5). Reagents were either purchased form Sigma-Aldrich (St.Louis, MO, USA), Fisher BioReagent (New Jersey, USA), Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Acetonitrile (HPLC grade; ≥99.8% purity) was purchased from EMD Chemicals Inc (New Jersey, USA).

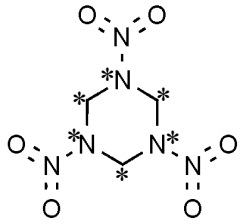

Figure 5. RDX structure (* position of heavy label, ^{13}C or ^{15}N)

4.2. Soil incubations

Soil samples used were either collected from agricultural sites or BTEX contaminated sites in Michigan. The agricultural sites had been previously amended with biosolids from a wastewater treatment plant, with the last application being within 1 to 4 years before sample collection. Soils were manually sorted, homogenized, air dried and sieved through a 4 mm screen after collection and stored at 4 °C until use (<1.5 years). In total, ten different soils were tested for RDX degradation under O_2 rich or depleted conditions. Test microcosms (triplicate autoclaved controls and live samples) were constructed with unlabeled RDX to determine RDX degradation potential. Stable isotope probing was conducted only on one soil (referred to as Soil 3).

Microcosms were constructed as previously described (Thompson et al., 2005b). Briefly, microcosms were assembled with soil (2 g; wet weight), a mineral salts medium (MSM), glucose (5.6 mM) and RDX (45 μM or 90 μM) and were incubated in the dark on a shaker. The MSM was prepared as previously described (Thompson et al., 2005b). Final masses (per liter) in each microcosm were as follows: KH_2PO_4, 0.218 g; K_2HPO_4, 0.278 g; $MgSO_4.7H_2O$, 0.16 mg; $FeSO_4.7H_2O$, 1.6 mg; $CaCl_2.2H_2O$, 0.024 mg; $MnCl_2.4H_2O$, 0.4 mg; H_3BO_3, 0.04 mg; $ZnCl_2$, 0.04 mg; $CuCl_2$, 0.024 mg; $Na_2MoO_4.2H_2O$, 0.008 mg; $CoCl_2.6H_2O$, 0.4 mg; $NiCl_2.6H_2O$ 0.04 mg; $Na_2MO_4.2H_2O$, 0.008 mg; $CoCl_2.6H_2O$, 0.4 mg; $NiCl_2.6H_2O$, 0.04 mg; and Na_2SeO_3, 0.4 mg.

Although all microcosms (50 mL or 160 mL) were closed with a rubber seal and aluminum crimp, a select number were also aerated between sampling days. All microcosms were briefly exposed to air during sampling, performed on day one and when RDX was expected to be removed completely (based on preliminary studies). Microcosms were prepared in duplicates or triplicates, covered in heavy-duty aluminum foil (to prevent RDX photo degradation) and were shaken at room temperature (~20 °C).

Microcosms for testing the extraction efficiency of RDX were constructed as described above. The SIP studies involved two different RDX concentrations (10 ppm and 20 ppm) in microcosms amended with labeled or unlabeled RDX as well as autoclaved controls.

4.3. RDX Extraction and Analysis

RDX extraction and analysis were as previously described (Thompson et al., 2005b). Briefly, sampling for RDX involved mixing and removal of 1 mL using a wide tip sterile serological pipette into a Nalgene Oak Ridge High-Speed FEP Centrifuge Tubes with Tefzel ETFE screw caps. RDX was extracted by adding equal volumes of acetonitrile and sonicating for 18 hours at 15 °C. The ultrasonic bath (Fischer Scienctific) was coil cooled by circulating cooled deionized water. At the end of 18 hours, the tubes were centrifuged, at 2900 rpm for 20 minutes. The supernatant (600 µL) was filtered using acetonitrile wetted filters (PVDF, 0.22 µm, Whattman). All samples were analyzed on the same day as extraction to minimize potential for RDX degradation.

HPLC analysis involved the following conditions and instrumentation: injector volume: 20 µL for samples and 10 µL for standards; isocratic 40% acetonitrile and 60% 0.1% H_3PO_4 acidified deionized water; mobile phase flow rate: 1 mL/min; Perkin Elmer series 200 autosampler; PE binary LC Pump 250; PE diode array detector 235C, wavelength 255 nm; column: Supelco Reverse Phase PAH C18 (25 cm X 4.6 mm, 5 µm).

4.4. SIP Studies: DNA Extraction and Ultracentrifugation

Following the complete removal of RDX, genomic soil DNA from the live labeled and unlabeled microcosms were extracted using the PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) as per manufacturer's instructions. Ultracentrifugation was performed in Quick – Seal Polyallomer tubes (Beckman Coulter) in a Thermo Sorvall WX ultra series centrifuge equipped with a step saver rotor system (70V6) for 46 hours at 178127 × *g* and 20 °C. All extracted DNA from a single microcosm (approximately 100 ng or more) was added to a Beckman Centrifuge tube along with a TE/CsCl solution. Buoyant densities (BD) were calculated by measuring the refractive index with a model AR200 digital hand-held refractometer (Leica Microsystems Inc.) before the tubes were sealed (Quick-Seal tube topper, Beckman Coulter). The initial buoyant density of the TE/CsCl solution was adjusted to 1.7828 g mL^{-1}, and that of the DNA and TE/CsCl solution to 1.7276 to 1.7285 g mL^{-1}

Following isopycnic gradient centrifugation, the DNA was divided into fractions (20-26 fractions) using a fractioning system (Beckman Coulter) and a syringe pump (Kd scientific). Deionized water was pumped into the top of the ultracentrifugation tubes and DNA-TE/CsCl mixture was collected from the bottom (heaviest DNA collected first) in volumes of 150 µL. The BD of each fraction was determined by measuring the refractive index with a model AR200 digital refractometer (Leica Microsystems, Inc.). The DNA was separated from the CsCl in each of the fractions by overnight glycogen-ethanol precipitation. The purified DNA was stored at -20 °C until further analysis.

4.5. SIP Studies: TRFLP and 16S rRNA Gene Sequencing

Heavy fractions (first 10 -12 fractions that had detectable DNA on 1% agarose gel) were analyzed by 16S rDNA terminal restriction fragment length polymorphism (TRFLP) using standard procedures (Liu et al., 1997). Universal primers 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG-3', 5' end-labeled with carboxyfluorescein) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Operon Biotechnologies) were utilized for PCR of all fractions. The PCR reaction mix included the following: 10 µL of template; 10 µL of 10X PCR buffer; 0.2mM of dNTP mix; 50 pmols of 27F-FAM; 50 pmols of 1492R; 2.5 units of Taq; and molecular biology grade water to a final volume of 100µL. The PCR program was: 94 °C (5 min); 94 °C (30 secs), 55 C (30 secs), 72 °C (1.5 min) (30 cycles); 72 °C (5 min). The PCR products (15 µL) were run on a 1% agarose gel and the first 10 to 12 heavy fractions illustrating a band on the gel were chosen for further analysis.

The PCR products were purified using a Qiagen PCR Purification kit following the manufacturer's instruction and concentrated in a 30 µL volume of elution buffer. An aliquot (13 µL) of the purified product (200 to 800 ng) was digested in a 15 µL digestion volume using 15 units of Hae III restriction enzyme (restriction site: CCGG). The digested DNA samples were analyzed in duplicates using Capillary Electrophoresis (ABi 3730 Genetic Analyzer, Research Technology Support Facility, Michigan State University). The percent abundance of fragments was determined using Genescan software.

Total DNA was PCR amplified (as described above with a 30 minutes extension step) and cloned into *Escherichia coli* TOPO 10 cells using TOPO TA cloning kit (Invitrogen Corporation). The *E. coli* cells were grown on LB broth (25 g L^{-1}) solidified with 15 g agar L^{-1} in the presence of 50 µg ampicillin mL^{-1} for 16 hours at 37°C. Individual colonies were isolated and grown in LB broth with ampicillin (50 µg mL^{-1}) for up to 16 hours and checked for growth. The clones with inserts were verified by PCR using M13 forward (5'-TGTAAAACGACGGCCAGT-3') and M13 reverse (5'-AACAGCTATGACCATG-3') primers and the plasmids were extracted using QIAPrep miniprep system (Qiagen, Inc.) and sequenced (using M13 forward and M13 reverse primers) at the Research Technology Support Facility at Michigan State University The Ribosomal Database Project's (Center for Microbial Ecology, Michigan State University) analysis tool called "Classifier" was used to assign taxonomic identity. The clustalW2 web tool (European Bioinformatics Institute) was utilized to align sequences.

4.6. *xplA* Functional gene

To determine if the functional gene *xplA* was present in soil 3 (soil used in SIP experiment), total genomic DNA extracted from RDX degrading microcosms was PCR amplified with *xlpA* specific forward (5′ GATGACCGCTGCTGCGTCCAT 3′) and reverse primers (5′ CCTGTTGCAGTCGCCTATACC 3′) (Indest et al., 2007). The PCR program was as follows: 94 °C (5 min); 94 °C (30 secs), 55 C (30 secs), 72 °C (1.5 min) (30 cycles); 72 °C (5 min). A positive control of *Rhodococcus rhodochrous* 11Y genomic DNA was included (supplied by Peter F. Andeer, University of Washington). The PCR samples were examined on a 1% agarose gel.

5.0. RESULTS AND DISCUSSION

5.1. Soil RDX Biodegradation

Only six out of the ten soils tested illustrated an ability to transform RDX (table 1). Little or no RDX removal was noted in the autoclaved controls suggesting a biological transformation mechanism.

Table 1. Experimental conditions and RDX degrading activity in soils tested.

Soil Description	Experimental Conditions	RDX degradation
Soil 1 (alfalfa field)	10 ppm RDX, not aerated	no
Soil 2 (corn field)	10 ppm RDX, not aerated	no
Soil 3 (soybean field)	10 ppm RDX, not aerated	yes
	10 ppm RDX, aerated	no
	20 ppm RDX, not aerated	yes
Soil 4 (BTEX site)	10 ppm RDX, not aerated	yes
	10 ppm RDX, aerated	no
Soil 5 (BTEX site)	10 ppm RDX, aerated	no
Soil 6 (corn field)	10 ppm RDX, aerated	no
Soil 7 (soybean field)	10 ppm RDX, not aerated	yes
Soil 8 (red kidney)	10 ppm RDX, not aerated	yes
Soil 9 (corn field)	10 ppm RDX, not aerated	yes
Soil 10 (corn field)	10 ppm RDX, not aerated	yes

The soils (soils 3, 4, 7, 8, 9 and 10) degraded RDX only when the microcosms were not aerated. For example, when soils 3 and 4 remained closed, RDX degradation was noted, however, when these microcosms were aerated daily, no transformation occurred (Figures 6 and 7). In soil 3, 10 ppm RDX was degraded in 14 days and 20 ppm RDX was degraded in ≤16 days. Soils 1, 2 and 6 showed no degradation under conditions tested. In contrast, soils 7, 8, 9 and 10 degraded ~10 ppm RDX in 16 days.

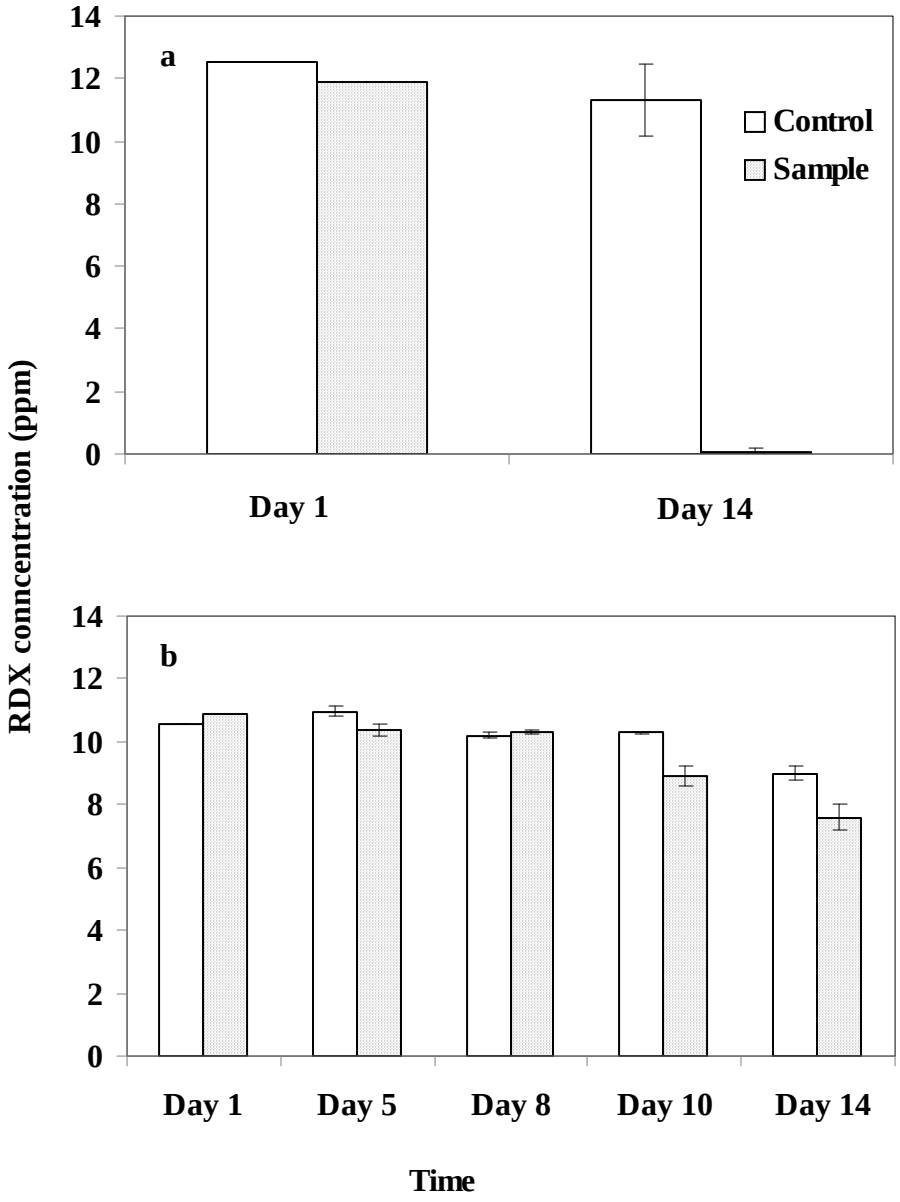

Figure 6. RDX removal in soil 3 in samples and control microcosms that remained closed (a) or were aerated daily (b) during the experimental period.

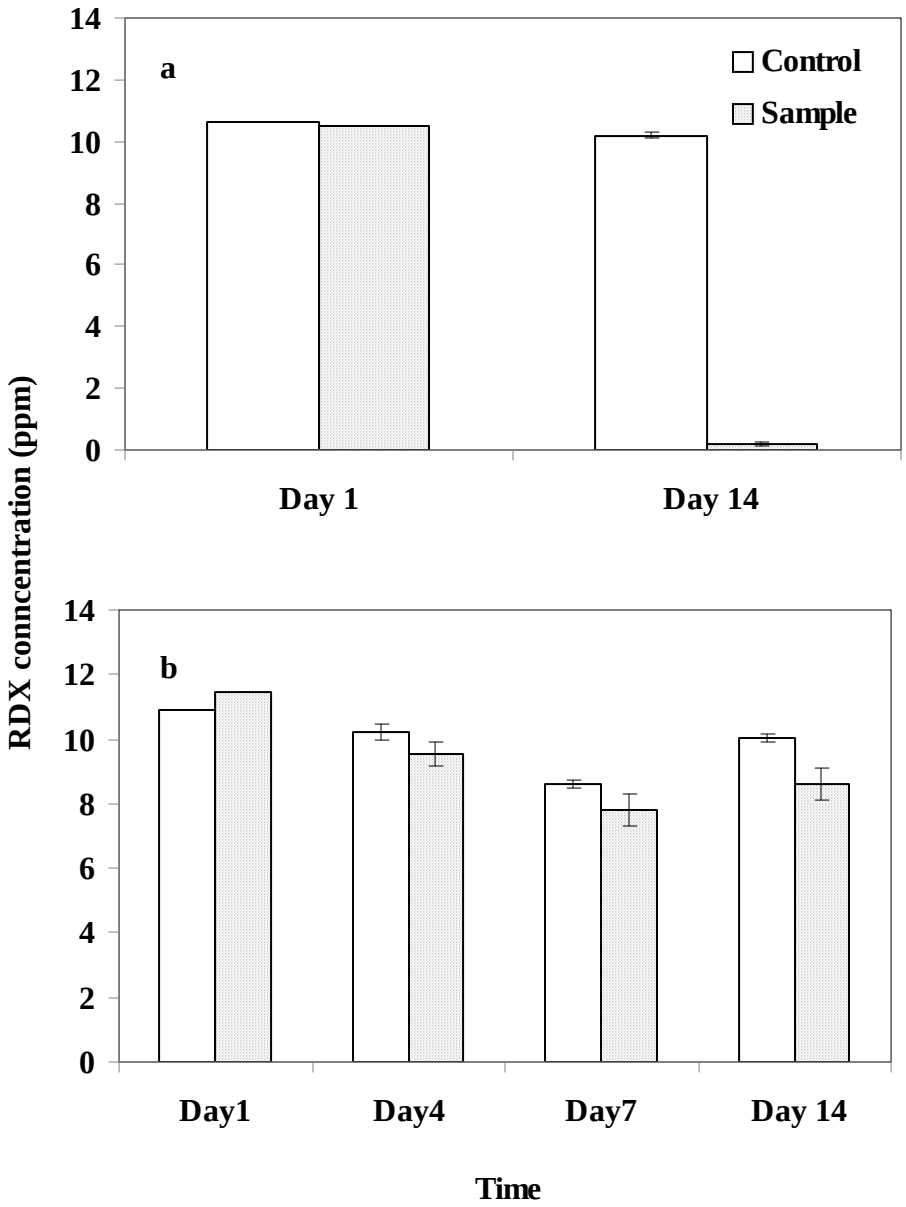

Figure 7. RDX removal in soil 4 in samples and control microcosms that remained closed (a) or were aerated daily (b) during the experimental period.

5.2. SIP Experiments

Following these preliminary RDX degradation screening experiments, SIP was conducted using microcosms constructed with soil 3 containing 10 ppm or 20 ppm RDX. The SIP studies involved microcosms amended with labeled or unlabeled RDX as well as autoclaved controls. After approximately two weeks (11 and 15 days for 10 and 20 ppm, respectively), RDX was below the detection level (<500 ppb) in all labeled and unlabeled sample microcosms, while no or little degradation was observed in the killed control samples (Figure 8).

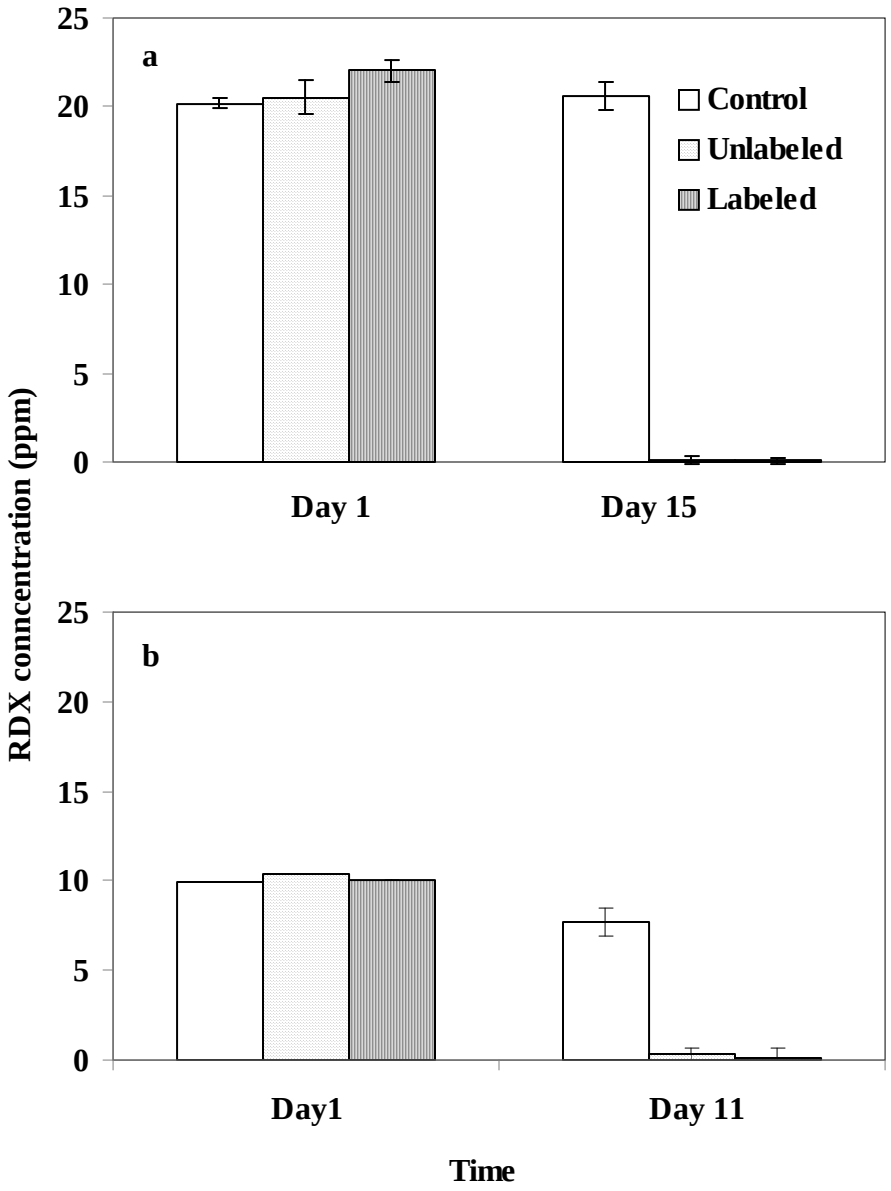

Figure 8. RDX removal in soil 3 in SIP samples (labeled and unlabeled) and control microcosms that remained closed at starting concentrations of 20 ppm (a) and 10 ppm (b).

Following RDX degradation DNA was extracted from the labeled and unlabeled RDX amended soil microcosms and was subject to ultracentrifugation, fractionation, followed by TRFLP analysis on the heaviest fractions. The measured buoyant density (BD) values for each fraction from each triplicate microcosm for both experiments (10 and 20 ppm RDX) illustrated the ultracentrifugation gradients were achieved and the DNA was therefore appropriately separated (Figures 9 and 10). For each triplicate, for both experiments, only the first (heaviest) 10 fractions containing detectable amplified DNA were subject to TRFLP analysis. The TRFLP data were then used to compare the relative abundance of each fragment in the heaviest fractions from the unlabeled RDX amended microcosms to the labelled RDX amended microcosms.

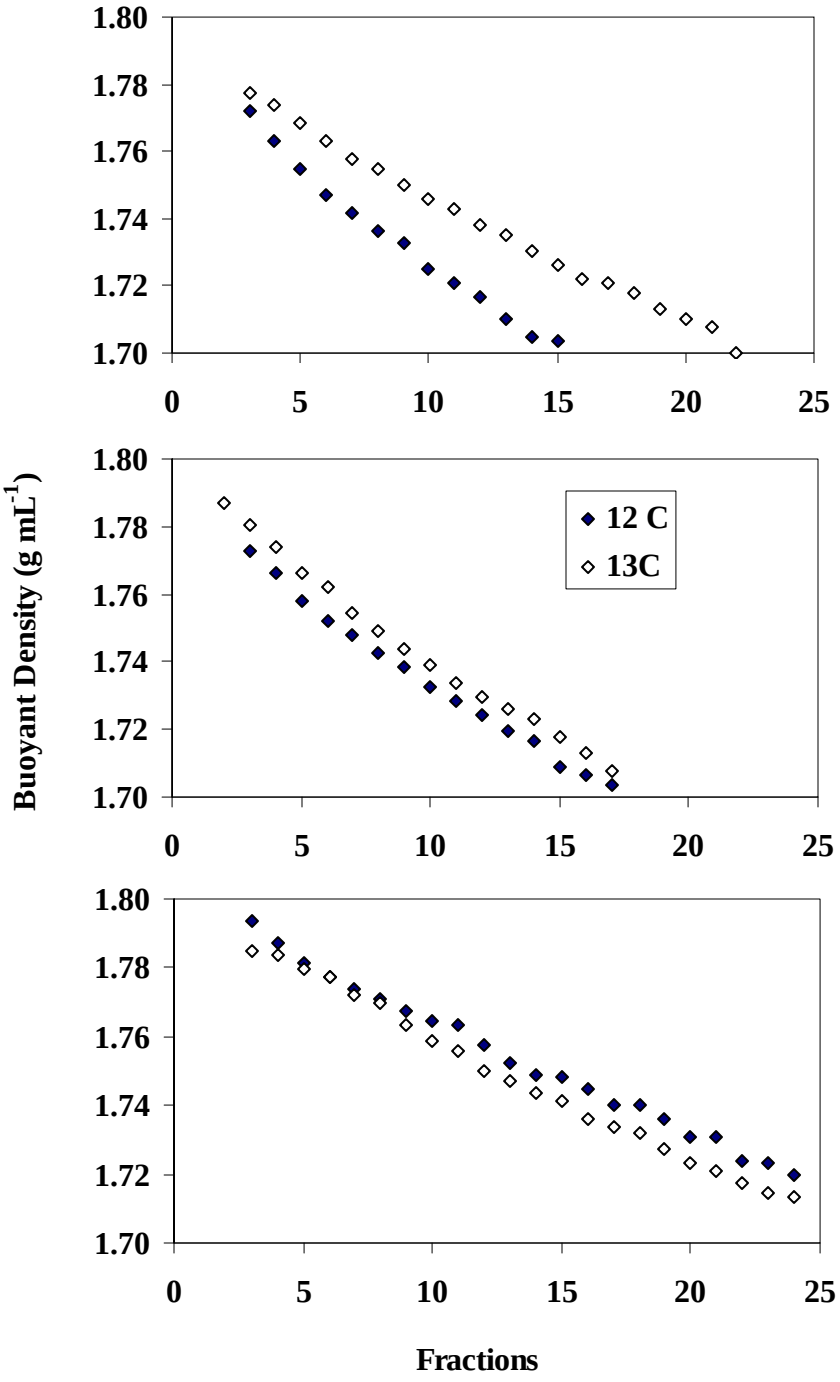

Figure 9. Buoyant density of each fraction from triplicate microcosms RDX amended with 10 ppm RDX.

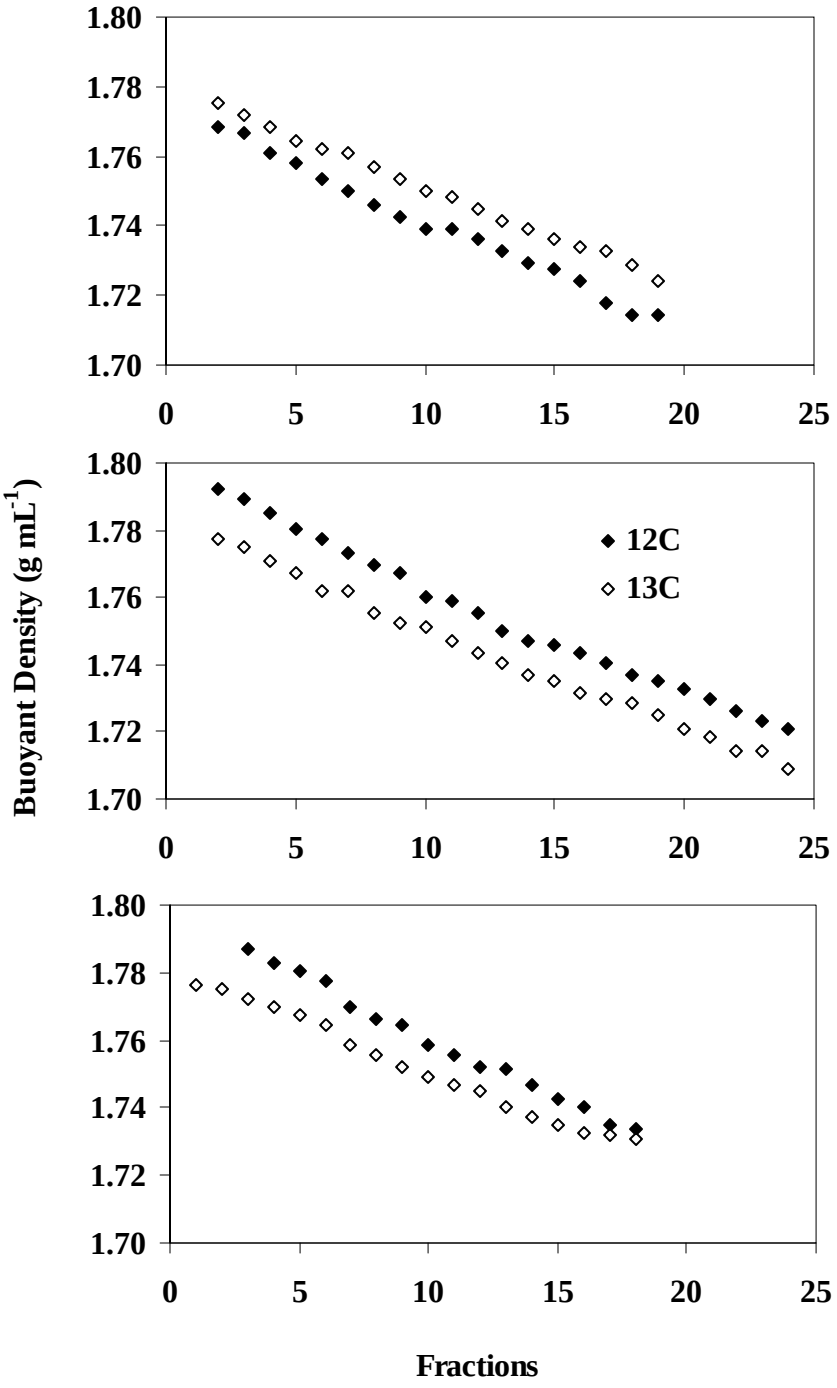

Figure 10. Buoyant density of each fraction from triplicate microcosms RDX amended with 20 ppm RDX.

The TRFLP data from the microcosms amended with 10 ppm did not show any significant differences between heavier labeled and unlabeled fractions in any of the triplicates. However, in the fractions from microcosms amended with a higher concentration of RDX (20 ppm), one TRFLP fragment (260 bp) showed a trend of label uptake in two of the three triplicates. Specifically, this fragment was of higher relative abundance in the heavier fractions from labeled samples when compared to the heavier fractions (of comparable BD) from the unlabeled samples (Figure 11). An example of the TRFLP profiles from a number of these fractions is provided. Specifically, a comparison of the TRFLP profiles from two of the heavy fractions from both labeled and unlabeled microcosms is shown (Figure 12). This comparison illustrates the trend apparent in other heavy fractions, that is, the dominance of fragment 260 bp in heavy labeled fractions compared to the heavy unlabeled fractions.

While many fragments were present in the heavier fractions of both the labeled and unlabeled samples, only fragments of size 260 bp showed a trend of increased relative abundance in heavier fractions from labeled samples when compared to the unlabeled treatments. The relative abundance of the other fragments tended to be similar in both treatments.

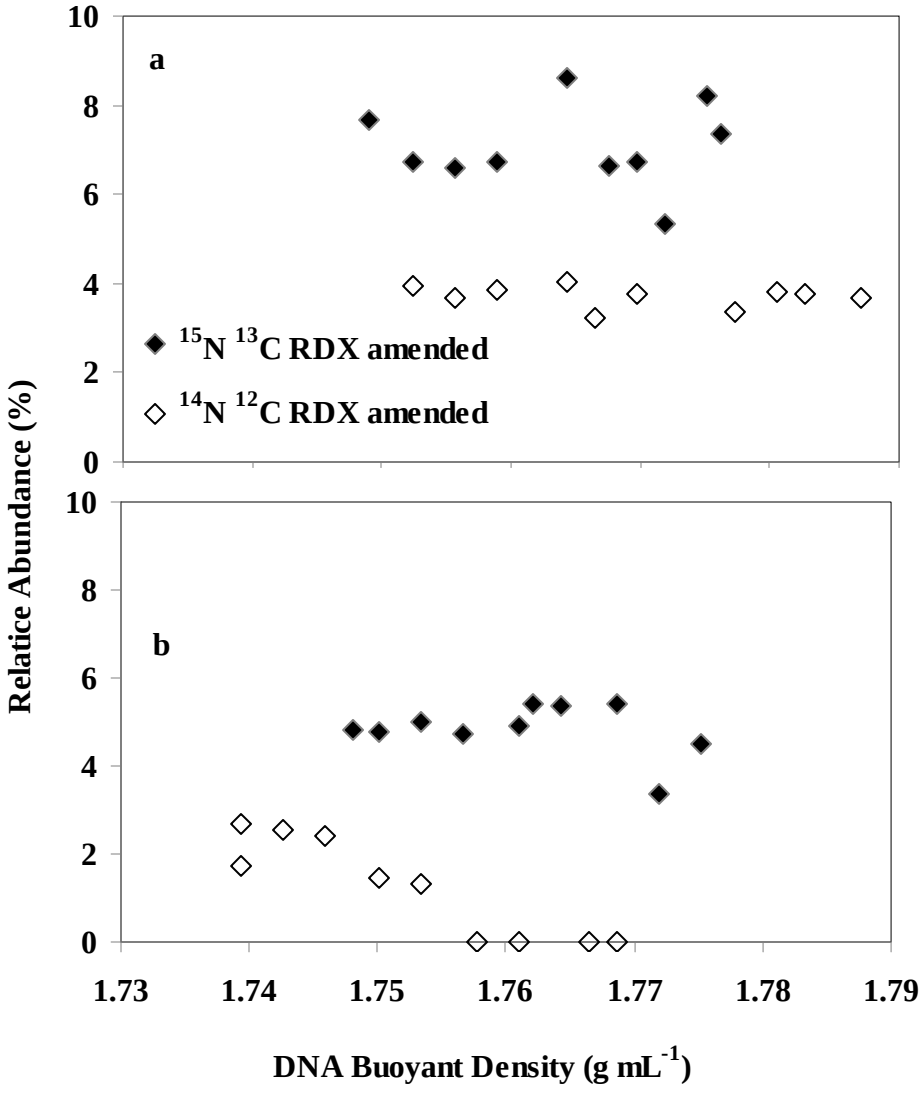

Figure 11. The TRFLP relative abundance (%) of fragment 260 bp in ultracentrifugation fractions with increasing buoyant density using DNA extracted from replicate microcosms sample (filled diamond, labelled RDX) and control microcosms (empty diamond, unlabelled RDX).

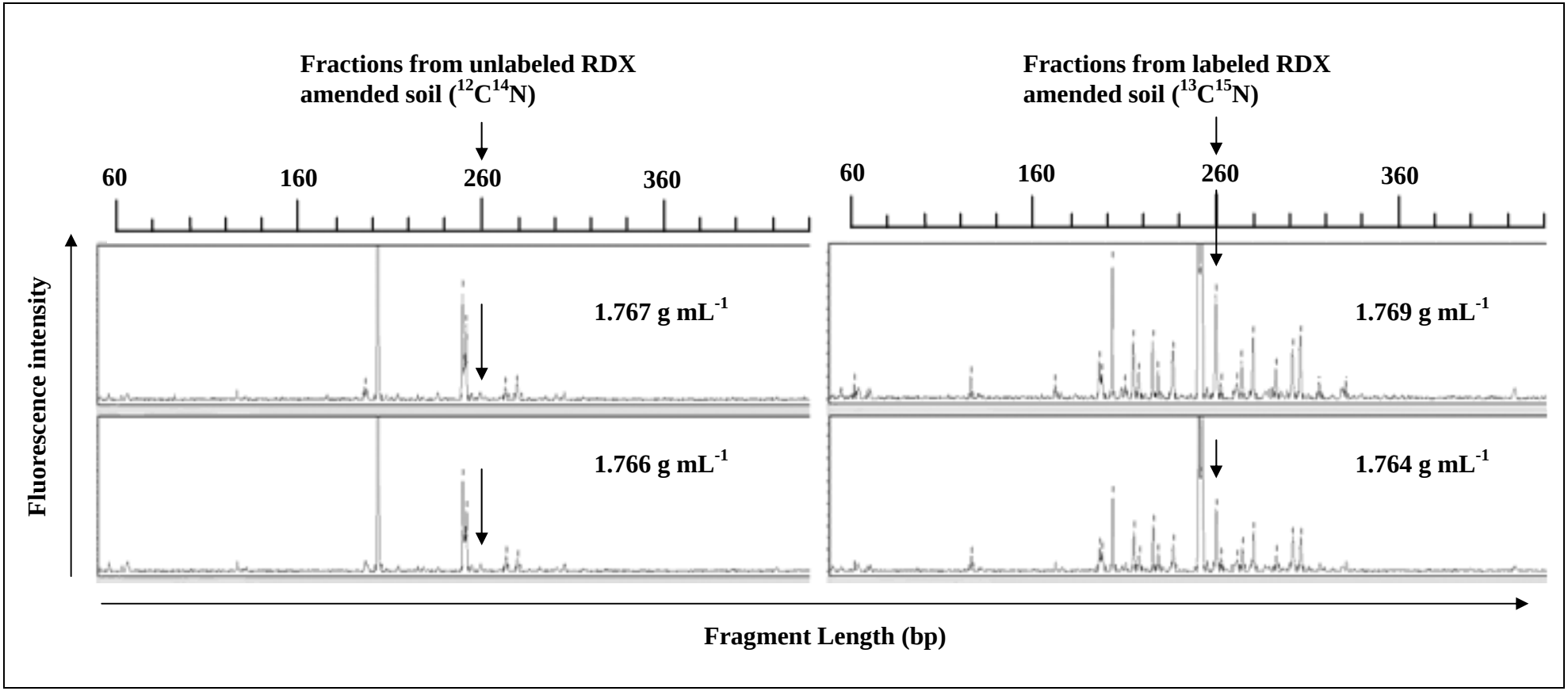

Figure 12. Comparison of the relative abundance of fragment 260 bp in heavy fraction TRFLP profiles obtained from labeled and unlabeled RDX amended samples.

5.3. 16S rRNA Gene Sequencing Results

Partial 16S rRNA gene sequences were obtained for 155 clones. The sequences, when classified using the "Classifier" within the Ribosomal Database Project (Michigan State University), belonged to *Actinobacteria*, *Acidobacteria*, *α-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria*, *Verrucomicrobiae*, *Gemmatimonadetes* and *Sphingobacteria*. The partial 16S rRNA gene sequences were virtually digested (restrictionmapper.org) with HaeIII enzyme, to identify clones that corresponded to the fragment of interest (260 bp). Of the 155 clones, 19 had terminal fragment lengths of 258-264 bp when virtually digested. This slight difference in the measured length of fragments with TRFLP (260 bp) and that predicted by sequence data (258-264 bp) has observed in other studies (Clement et al., 1998; Liu et al., 1997). The analysis of these partial 16S rRNA sequences indicated the organism responsible for RDX degradation (260 bp) belongs to either the *Sphingobacteria* (18 clones) or the *Acidobacteria* (1 clone). The closest relatives of these 19 clones were determined using BLAST in the Genbank database (table 2).

Table 2. The closest matches between the 19 partial 16S rRNA gene sequences identified here containing a 260 bp TRFLP fragment and those present in the Genbank database.

	Genbank Accession Number and Description	**Environmental Sample**	**Similarity**
1	EF605989 - Unidentified bacterium clone 44 C1 RHIZO T7s	Soil, arable land	708/717 (98%)
2, 3	EU589295 - Uncultured soil bacterium clone 2 C12	Chinese rice paddy field soil	816/825 (98%)
4	EU979037 - Uncultured *Bacteroidetes* bacterium clone g28	Rhizosphere	757/789 (95%)
5	EU160125 - Uncultured bacterium clone 2N6-113	Rhizosphere	729/753 (96%),
6, 10	FJ801203 - Uncultured bacterium clone ZWB4-5	Wetland water	746/748 (99%)
7, 8	FJ612391 - Uncultured bacterium clone DP10.3.63	Lake ecosystem	703/751 (93%
9	DQ378273 - Uncultured soil bacterium clone M60 Pitesti	Oil polluted soil	727/749 (97%)
11	AY921683 - Uncultured *Bacteroidetes* bacterium clone AKYG1587	Farm soil	783/792 (98%)
12	EF018642 - Uncultured *Bacteroidetes* bacterium clone Amb 16S 923	Soil, Aspen	727/752 (96%)
13	AB241539 - Uncultured bacterium	Rhizosphere Phragmites	816/825 (98%)
14, 17	EF393429 - Uncultured bacterium clone ORSFC2 e12	Ohio River Sediments	726/735 (98%
15, 16, 18	DQ444038 - Uncultured bacterium clone PH10-1	River sediments	566/626 (90%)
19	EU122748 - Uncultured *Acidobacteria* bacterium clone KL2-001	TNT contaminated soil	635/645 (98%),

5.4. *xplA* Results

Although the positive control (*Rhodococcus rhodochrous* 11Y genomic DNA) produced a PCR product, the soil genomic DNA did not (soil 3), indicating it is likely not important for RDX degradation in the microcosms constructed from soil 3.

5.5. Identified RDX Degrading Microorganisms

Biodegradation of RDX has been reported both under aerobic and anaerobic conditions. However, for all soils studied here, RDX was degraded only in the absence of oxygen. Previously reported bacterial strains that have illustrated anaerobic RDX degrading abilities in pure culture include the following: *Acetobacterium plausodum, Acetobacterium malicum, Clostridium acetobutylicum, Clostridium kluyveri, Clostridium bifermentans, Clostridium celerecrescens, Clostridium saccharolyticum, Clostridium butyricum, Citrobacter freundii NS2, Desulfovibrio desulfuricans, Enterobacer cloacae, Klebsiella pneumoniae SCZ-1, Morganella morganii B2, Providencia* rettgeri B1 and *Serratia marcescens* (Fuller and Steffan, 2008). In the current study, RDX degradation was linked to the class *Sphingobacteria* (phylum *Bacteroidetes*) as well as the phylum *Acidobacteria*. To our knowledge, neither have previously been linked to RDX biodegradation.

Although not previously linked to RDX biodegradation, *Sphingobacteria* have been identified (through 16S rRNA gene sequencing) in various organic contaminant-degrading mixed culture samples or contaminated sites. Both patterns suggest these organisms may be responsible for the biodegradation of a number of environmental contaminants. For example, this group was found in groundwater at a trichloroethene contaminated site (Macbeth et al., 2004). In addition, *Sphingobacteria* groups were identified in PAH (benzo(a)anthracene and chrysene) degrading soil slurry microcosms (Llado et al., 2009). *Sphingobacteria* have also been identified in microcosms transforming PCBs (Luo et al., 2008). Further, the phylogenetic analysis of a membrane bioreactor treating nitrate (NO_3^-) and perchlorate (ClO_4^-) indicated that the nitrate reducing bacteria of the bacterial classes *γ-Proteobacteria* and *Sphingobacteria* were the metabolically dominant members within the stabilized biofilm (Sahu et al., 2009). Although these studies do not provide a direct link between *Sphingobacteria* and contaminant transformation, they indicate these organisms are likely important community members for these processes and could suggest a wide substrate range.

The other RDX degrading microorganism classified within the phylum *Acidobacteria*. To our knowledge, organisms within this phylum have not previously been linked to RDX degradation. In fact, although *Acidobacteria* are widespread and abundant in soils, little is known about these organisms (Zhang and Xu, 2008). The phylum was first identified in 1997 (Ludwig et al., 1997), contains a number of soil isolates obtained with various new cultivation strategies (Joseph et al., 2003; Sait et al., 2002; Stevenson et al., 2004) as well as seven species with validly published names (*Acidobacterium capsulatum*, *Holophaga foetida*, *Geothrix fermentans*, *Terriglobus roseus*, *Edaphobacter modestus*, *Edaphobacter aggregans, Acanthopleuribacter pedis*) (Coates et al., 1999; Eichorst et al., 2007; Fukunaga et al., 2008; Kishimoto et al., 1991; Koch et al., 2008; Liesack et al., 1994). However, the vast majority of *Acidobacteria* for which 16S rRNA gene sequences have been obtained still remain uncultured and their role in the environment is poorly understood (Meisinger et al., 2007). Interestingly, based on 16S rRNA clone libraries, it appears that members of this phylum typically represent a significant portion (~20%, but up to

80%) of soil bacterial communities (Chan et al., 2006; Dunbar et al., 1999; Janssen, 2006; Lee et al., 2008). Further, these organisms appear to be genetically diverse, with the branching depth being nearly as great as the *Proteobacteria* phylum (Hugenholtz et al., 1998). In addition, they are also metabolically diverse, with 16S rRNA genes being found in a wide range of environmental samples including a deep sea ecosystem (Quaiser et al., 2008), uranium contaminated subsurface sediments (Barns et al., 2007), chromium contaminated river system (Branco et al., 2005) an acidic mining lake (Kleinsteuber et al., 2008), a lead-zinc mine tailing site (Mendez et al., 2008) and wastewater treatment systems (Crocetti et al., 2002).

5.6. Other Studies using SIP to Identify RDX Degrading Microorganisms

Since the initiation of the current project, there has been one report of the application of SIP to investigate RDX degraders *in situ* (Roh et al., 2009). In that study, RDX degradation was examined in groundwater microcosms amended with different nutrient sources and ring ^{15}N labelled RDX was used. The authors reported fifteen 16S rRNA gene sequences were associated with RDX transformation. These included bacteria belonging to *Actinobacteria* (two clones), α-*Proteobacteria* (seven clones), and γ-*Proteobacteria* (six clones). The authors found five sequences with high similarity to known RDX degraders (*Enterobacter cloacae* and *Pseudomonas fluorescens* I-C). In addition, they detected six sequences of the RDX degrading gene, *xplA*. This recent report provides additional evidence of the utility of SIP for understanding RDX degradation in mixed culture samples.

5.7. Implication and Relevance towards Project Objectives

The current project illustrates the importance of SIP for discovering unknown RDX degraders. Both the current project and the RDX SIP project described above indicate SIP is a powerful method for understanding RDX degradation in complex, mixed culture samples. These data can then be used for biomarker development to investigate and gain a better understanding RDX removal at contaminated sites.

6.0. CONCLUSIONS AND IMPLICATIONS FOR FUTURE WORK

Several conclusions can be drawn from the current study. First, even though the soils tested (agricultural and BTEX contaminated sites soils) had not been previously exposed to RDX, six out of the ten soils demonstrated an ability to transform RDX. These data indicate RDX degrading species may be more common than previously thought. Significantly, RDX degradation only occurred under oxygen depleted conditions. Second, the application of SIP enabled the identification of bacteria linked to RDX biodegradation in complex, mixed culture samples. Third, the bacteria identified (*Sphingobacteria* and *Acidobacteria*) did not have previous links to RDX degradation, indicating the discovery of novel RDX degraders. Finally, these sequence data provide an additional source for biomarker development for use at contaminated sites for assessing RDX natural attenuation potential. In summary, the proof-of-concept that SIP could be used to identify *in situ* RDX degraders in complex, mixed culture samples was proven successful.

The current study highlighted several limitations to this approach, including a lack of SIP signal at the lower RDX concentration (10 ppm). Further, the increase in DNA BD was small and did not facilitate SIP over time to investigate cross feeding. Finally, significant time was needed to find the SIP signal, involving the production and TRFLP analysis of precise gradient fractions in triplicate samples. However, these methods have been developed and can be applied with ease to other mixed cultures or environmental samples.

Future research would involve the following objectives:

- **Objective 1.** Design specific 16S rRNA gene primers for the novel RDX degraders (*Sphingobacteria* and *Acidobacteria*) identified here.

- **Objective 2.** Apply the developed SIP approach to the five other RDX degrading microcosms; all constructed from different soil sources (soils 4, 7, 8, 9 & 10) and likely to contain a diverse set of RDX degraders.

- **Objective 3.** Obtain samples from RDX contaminated sites for SIP analysis.

- **Objective 4.** Develop a library of biomarkers from the above SIP studies (objectives 2 and 3).

- **Objective 5.** Test a large number of environmental samples for the presence of these species and correlate this to RDX degradation rates.

The current and proposed work is important because known RDX degrading species and species from RDX enrichments often have not been found at field sites where RDX is being degraded. Therefore, it is not clear which pathways are most important at contaminated sites. The current work identified previously undiscovered RDX degraders and therefore contaminated sites can now also be probed for these species. The extension of this work to other soil sources will further extend this library of *in situ* RDX degraders for their use as biomarkers.

7.0. LITERATURE CITED

Adrian, N.R., Chow, T., 2001. Identification of hydroxylamino-dinitroso-1,3,5-triazine as a transient intermediate formed during the anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Environmental Toxicology and Chemistry 20,1874-1877.

Adrian, N.R., Arnett, C.M., 2004. Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by Acetobacterium malicum strain HAAP-1 isolated from a methanogenic mixed culture. Current Microbiology 48,332-340.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiological Reviews 59,143-169.

Barns, S.M., Cain, E.C., Sommerville, L., Kuske, C.R., 2007. *Acidobactetia* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Applied and Environmental Microbiology 73,3113-3116.

Beller, H.R., 2002. Anaerobic biotransformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by aquifer bacteria using hydrogen as the sole electron donor. Water Research 36,2533-2540.

Bhushan, B., Trott, S., Spain, J.C., Halasz, A., Paquet, L., Hawari, M., 2003. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a rabbit liver cytochrome p450: insight into the mechanism of RDX biodegradation by *Rhodococcus* sp strain DN22. Applied and Environmental Microbiology 69,1347-1351.

Binks, P.R., Nicklin, S., Bruce, N.C., 1995. Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas-Maltophilia* Pb1. Applied and Environmental Microbiology 61,1318-1322.

Boopathy, R., Gurgas, M., Ullian, J., Manning, J.F., 1998. Metabolism of explosive compounds by sulfate-reducing bacteria. Current Microbiology 37,127-131.

Branco, R., Chung, A.P., Verissimo, A., Morais, P.V., 2005. Impact of chromium-contaminated wastewaters on the microbial community of a river. FEMS Microbiology Ecology 54,35-46.

Chan, O.C., Yang, X.D., Fu, Y., Feng, Z.L., Sha, L.Q., Casper, P., Zou, X.M., 2006. 16S rRNA gene analyses of bacterial community structures in the soils of evergreen broad-leaved forests in south-west China. Fems Microbiology Ecology 58,247-259.

Clement, B.G., Kehl, L.E., DeBord, K.L., Kitts, C.L., 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. Journal of Microbiological Methods 31,135-142.

Coates, J.D., Ellis, D.J., Gaw, C.V., Lovley, D.R., 1999. *Geothrix fermentans* gen. nov., sp nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. International Journal of Systematic Bacteriology 49,1615-1622.

Coleman, N.V., Nelson, D.R., Duxbury, T., 1998. Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. Soil Biology & Biochemistry 30,1159-1167.

Coleman, N.V., Spain, J.C., Duxbury, T., 2002. Evidence that RDX biodegradation by *Rhodococcus* strain DN22 is plasmid-borne and involves a cytochrome p-450. Journal of Applied Microbiology 93,463-472.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L., Blackall, L.L., 2002. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. Microbiology-Sgm 148,3353-3364.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., Kuske, C.R., 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Applied and Environmental Microbiology 65,1662-1669.

Eichorst, S.A., Breznak, J.A., Schmidt, T.M., 2007. Isolation and characterization of soil bacteria that define *Teniglobus* gen. nov., in the phylum *Acidobacteria*. Applied and Environmental Microbiology 73,2708-2717.

Fournier, D., Trott, S., Hawari, J., Spain, J., 2005. Metabolism of the aliphatic nitramine 4-nitro-2,4-diazabutanal by Methylobacterium sp strain JS178. Applied and Environmental Microbiology 71,4199-4202.

Fournier, D., Halasz, A., Spain, J., Fiurasek, P., Hawari, J., 2002. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22. Applied and Environmental Microbiology 68,166-172.

Fournier, D., Halasz, A., Spain, J., Spanggord, R.J., Bottaro, J.C., Hawari, J., 2004. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by Phanerochaete chrysospotium. Applied and Environmental Microbiology 70,1123-1128.

Fukunaga, Y., Kurahashi, M., Yanagi, K., Yokota, A., Harayama, S., 2008. *Acanthopleuribacter pedis* gen. nov., sp nov., a marine bacterium isolated from a chiton, and description of *Acanthopleuribacteraceae* fam.nov., *Acanthopleuribacterales* ord. nov., *Holophagaceae* fam. nov., *Holophagales* ord. nov and *Holophagae* classis nov in the phylum '*Acidobacteria*'. International Journal of Systematic and Evolutionary Microbiology 58,2597-2601.

Fuller, M.E., Steffan, R.J., 2008. Groundwater chemistry and microbial ecology effects on explosives biodegradation. SERDP Final Report Project ER-1378.

Hawari, J., Beaudet, S., Halasz, A., Thiboutot, S., Ampleman, G., 2000a. Microbial degradation of explosives: biotransformation versus mineralization. Applied Microbiology and Biotechnology 54,605-618.

Hawari, J., Halasz, A., Sheremata, T., Beaudet, S., Groom, C., Paquet, L., Rhofir, C., Ampleman, G., Thiboutot, S., 2000b. Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. Applied and Environmental Microbiology 66,2652-2657.

Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity (vol 180, pg 4765, 1998). Journal of Bacteriology 180,6793-6793.

Indest, K.J., Crocker, F.H., Athow, R., 2007. A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene. Journal of Microbiological Methods 68,267-274.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied and Environmental Microbiology 72,1719-1728.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., Janssen, P.H., 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Applied and Environmental Microbiology 69,7210-7215.

Kishimoto, N., Kosako, Y., Tano, T., 1991. *Acidobacterium capsulatum* gen. nov., sp. nov.: an acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. Current Microbiology 22,1-7.

Kitts, C.L., Cunningham, D.P., Unkefer, P.J., 1994. Isolation of 3 hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family *Enterobacteriaceae* from nitramine explosive-contaminated soil. Applied and Environmental Microbiology 60,4608-4611.

Kitts, C.L., Green, C.E., Otley, R.A., Alvarez, M.A., Unkefer, P.J., 2000. Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Canadian Journal of Microbiology 46,278-282.

Kleinsteuber, S., Muller, F.D., Chatzinotas, A., Wendt-Potthoff, K., Harms, H., 2008. Diversity and *in situ* quantification of *Acidobacteria* subdivision 1 in an acidic mining lake. Fems Microbiology Ecology 63,107-117.

Koch, I.H., Gich, F., Dunfield, P.F., Overmann, J., 2008. *Edaphobacter modestus* gen. nov., sp nov., and *Edaphobacter aggregans* sp nov., acidobacteria isolated from alpine and forest soils. International Journal of Systematic and Evolutionary Microbiology 58,1114-1122.

Lee, S.H., Ka, J.O., Cho, J.C., 2008. Members of the phylum *Acidobacteria* are dominant and metabolically active in rhizosphere soil. FEMS Microbiology Letters 285,263-269.

Liesack, W., Bak, F., Kreft, J.U., Stackebrandt, E., 1994. *Holophaga foetida* gen-nov, sp-nov, a new, homoacetogenic bacterium degrading methoxylated aromatic compounds. Archives of Microbiology 162,85-90.

Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J., 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology 63,4516-4522.

Llado, S., Jimenez, N., Vinas, M., Solanas, A.M., 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation 20,593-601.

Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S., Hartmann, A., Schleifer, K.H., 1997. Detection and in situ identification of representatives of a widely distributed new bacterial phylum. Fems Microbiology Letters 153,181-190.

Luo, W., D'Angelo, E.M., Coyne, M.S., 2008. Organic carbon effects on aerobic polychlorinated biphenyl removal and bacterial community composition in soils and sediments. Chemosphere 70,364-373.

Macbeth, T.W., Cummings, D.E., Spring, S., Petzke, L.M., Sorenson, K.S., 2004. Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. Applied and Environmental Microbiology 70,7329-7341.

McCormick, N.G., Cornell, J.H., Kaplan, A.M., 1981. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Applied and Environmental Microbiology 42,817-823.

Meisinger, D.B., Zimmermann, J., Ludwig, W., Schleifer, K.H., Wanner, G., Schmid, M., Bennett, P.C., Engel, A.S., Lee, N.M., 2007. In situ detection of novel *Acidobacteria* in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA). Environmental Microbiology 9,1523-1534.

Mendez, M.O., Neilson, J.W., Maier, R.M., 2008. Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. Applied and Environmental Microbiology 74,3899-3907.

Quaiser, A., Lopez-Garcia, P., Zivanovic, Y., Henn, M.R., Rodriguez-Valera, F., Moreira, D., 2008. Comparative analysis of genome fragments of *Acidobacteria* from deep Mediterranean plankton. Environmental Microbiology 10,2704-2717.

Roh, H., Yu, C.P., Fuller, M.E., Chu, K.H., 2009. Identification of Hexahydro-1,3,5-trinitro-1,3,5-triazine-Degrading Microorganisms via N-15-Stable Isotope Probing. Environmental Science & Technology 43,2505-2511.

Rylott, E.L., Jackson, R.G., Edwards, J., Womack, G.L., Seth-Smith, H.M.B., Rathbone, D.A., Strand, S.E., Bruce, N.C., 2006. An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX. Nature Biotechnology 24,216-219.

Sahu, A.K., Conneely, T., Nusslein, K., Ergas, S.J., 2009. Hydrogenotrophic denitrification and perchlorate reduction in ion exchange brines using membrane biofilm reactors. Biotechnology and Bioengineering 104,483-491.

Sait, M., Hugenholtz, P., Janssen, P.H., 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environmental Microbiology 4,654-666.

Seth-Smith, H.M.B., Rosser, S.J., Basran, A., Travis, E.R., Dabbs, E.R., Nicklin, S., Bruce, N.C., 2002. Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from *Rhodococcus rhodochrous*. Applied and Environmental Microbiology 68,4764-4771.

Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., Breznak, J.A., 2004. New strategies for cultivation and detection of previously uncultured microbes. Applied and Environmental Microbiology 70,4748-4755.

Thompson, K.T., Crocker, F.H., Fredrickson, H.L., 2005a. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. Applied and Environmental Microbiology 71,8265-8272.

Thompson, K.T., Crocker, F.H., Fredrickson, H.L., 2005b. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp. Applied and Environmental Microbiology 71,8265-8272.

Young, D.M., Unkefer, P.J., Ogden, K.L., 1997. Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a prospective consortium and its most effective isolate *Serratia marcescens*. Biotechnology and Bioengineering 53,515-522.

Zhang, L., Xu, Z.H., 2008. Assessing bacterial diversity in soil. Journal of Soils and Sediments 8,379-388.

Zhao, J.S., Spain, J., Hawari, M., 2003a. Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source. FEMS Microbiology Ecology 46,189-196.

Zhao, J.S., Paquet, L., Halasz, A., Hawari, J., 2003b. Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in Clostridium bifermentans HAW-1. Applied Microbiology and Biotechnology 63,187-193.

Zhao, J.S., Halasz, A., Paquet, L., Beaulieu, C., Hawari, J., 2002. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. Applied and Environmental Microbiology 68,5336-5341.

Zhao, J.S., Greer, C.W., Thiboutot, S., Ampleman, G., Hawari, J., 2004a. Biodegradation of the nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions. Canadian Journal of Microbiology 50,91-96.

Zhao, J.S., Spain, J., Thiboutot, S., Ampleman, G., Greer, C., Hawari, J., 2004b. Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiology Ecology 49,349-357.